Carbon conversion efficiency and ^{13}C labeling of the tricarboxylic acid cycle in lesquerella (*Physaria fendleri*) embryos

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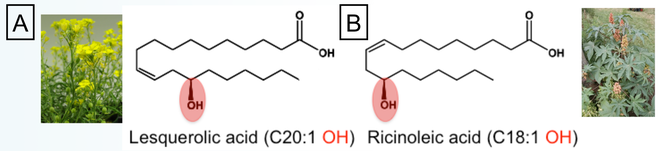
BACKGROUND

Lesquerella (*Physaria fendleri*) is a Brassicaceae that produces a valuable class of compounds in its embryos called hydroxy fatty acids. These fatty acids are used widely in industry to produce cosmetics, coatings, greases, plastics, paints, and biofuel, among others. The current source of hydroxy fatty acids is ricinoleic acid from the castor plant (Ricinus communis), which also produces the highly toxic compound ricin that has eliminated castor plant cultivation in the United States. Lesquerella produces lesquerolic acid, a hydroxy fatty acid with only two additional carbons as compared to ricinoleic acid, which therefore performs in a chemically similar manner. (Figure 1).



Lesquerolic acid (C20:1 OH) Ricinoleic acid (C18:1 OH)

Figure 1. Hydroxy fatty acids in lesquerella (A) and Castor (B) plants.

Since it is non-toxic and already grows in the wild in the southwest US as a winter annual that will not compete with food crops, lesquerella could serve as a suitable alternative to castor oil. However to become economically viable, lesquerella first must be engineered to produce more of these fatty acids to meet demands. The most recent work done towards meeting this end goal is: (i) quantifying the development of the biomass components of lesquerella embryos in planta (fatty acids, starch, protein, and cell wall); (ii) identifying culture conditions for growing embryos in vitro so that their biomass composition matches that of embryos grown in planta; (iii) calculating the rates of substrate consumption by embryos; (iv) determining the efficiency at which carbon is converted into biomass; and (v) determine the mass isotopomer abundance within the tricarboxylic acid cycle of ^{13}C-labeled embryos.

HYPOTHESIS

Lesquerella embryos can be cultured *in vivo* to mimick *in planta* growth and this culturing medium can be used to label embryos for beginning ^{13}C-flux analysis.

BIOMASS COMPOSITION

Culture conditions for labeling lesquerella embryos

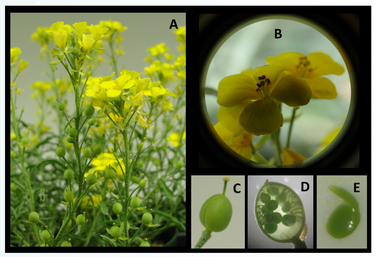
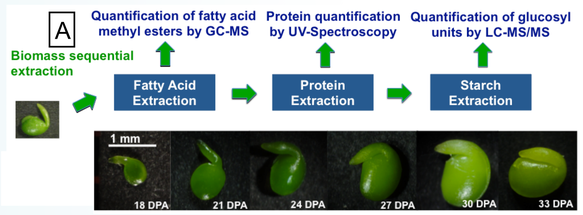


Figure 2. Anatomy of lesquerella plants. Lesquerella plants were grown at 22°C with 16/8-h light/dark cycle in a growth chamber. **(A)** Mature plant; **(B)** Flowers; **(C, D)** Pod containing seeds; **(E)** Embryo 21 days post anthesis (DPA).



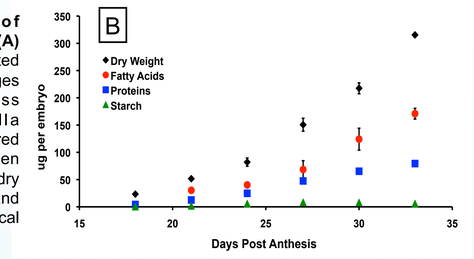


Figure 3. Development of lesquerella embryos *in planta*. (A) Biomass components were extracted from lesquerella at 6 different stages of development. **(B)** Biomass accumulation in lesquerella embryos. The black diamonds, red circles, blue squares and green triangles are respectively, the dry weight, the fatty acid, protein, and starch contents (n = 3 biological replicates) (Cocuron et al., 2014).

A lesquerella embryo grew on average 19.3 µg DW.day^{-1} (R^2 = 0.95), accumulating fatty acids, proteins, and starch at rates of 11.0 (R^2 = 0.94), 5.3 µg.day^{-1} (R^2 = 0.98), and 0.4 µg.day^{-1} (R^2 = 0.97), respectively (Figure 5.B).

METHODS

Culture conditions for labeling lesquerella embryos

Endosperm, the source that feeds the embryo, was extracted from seeds and analyzed by LC/MS-MS. Glucose and sucrose were the main sources of carbon-based biomass components and glutamine was the main source of nitrogen.

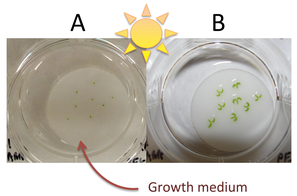


Figure 4. (A) 18 DAP embryos at day 0 of culture. Developing embryos were dissected at 18 DAP under aseptic conditions and transferred into six-well petri-dishes, on double-glass filters to avoid anoxia. The glass filters were soaked with 1 mL of culture medium. The six-well petri-dishes were incubated at 22°C under 12 µE of light intensity. **(B) 27 DAP embryos after 9 days of culture.** Picture of the same embryos after 9 days of culture in the conditions described above.

RESULTS

Culture conditions for labeling lesquerella embryos

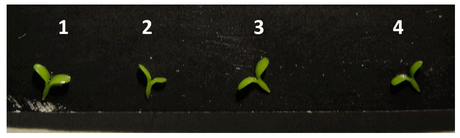


Figure 5. Isolated embryos after 9 days of culture. Embryos after growing in the successful medium for 9 days (27 DAP) as described in Figure 4. Each embryo numbered 1-4 was taken from a well as seen in Figure 6B for a total of four biological replicates.

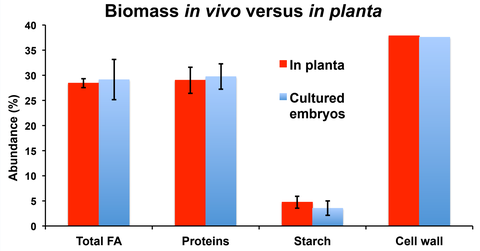


Figure 6. Biomass *in vivo* versus *in planta*. Biomass for cultured embryos was determined by the previously described methods.

Embryos grown in the medium that is used show a biomass composition that matches that of embryos grown *in planta*. This medium will use ^{13}C-labeled glucose and glutamine to follow labeling in the central metabolism of the lesquerella embryos.

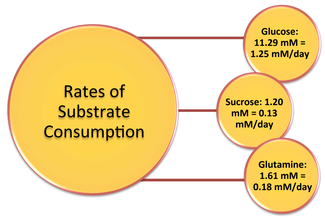


Figure 7. Rates of substrate uptake. Leftover media glucose, sucrose, and glutamine content was measured by LC-MS/MS in reference to a ^{13}C standard and compared to the initial media substrate composition.

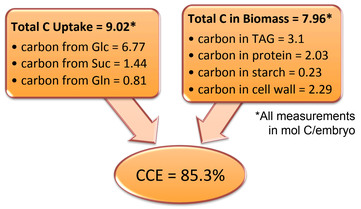


Figure 8. Carbon Conversion Efficiency. Carbon input from glucose, sucrose, and glutamine as measured by rates of substrate consumption were compared to carbon output in the form of quantified biomass components.

Acknowledgements & References

• **Research support was provided by state funds appropriated to the Ohio Plant Biotechnology Consortium through The Ohio State University, Ohio Agricultural Research and Development Center.**

• **Targeted Metabolomics Laboratory (metabolomics.osu.edu)**

• **Cocuron, JC, Anderson, B, Boyd, A & Alonso, AP (2014). Targeted metabolomics of *Physaria fendleri*, an industrial crop producing hydroxy fatty acids. *Plant and Cell Physiology* 55:3 620-633.**

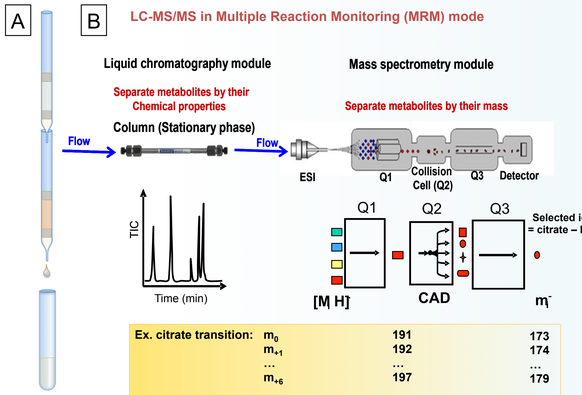
• **Alonso AP, Goffman F, Ohlrogge JB, Sachar-Hill Y (2007). Carbon conversion efficiency and central metabolic fluxes in developing sunflower (*Helianthus annuus* L.) embryos. *The Plant Journal* 52:2 296-308.**

METHODS

Following labeling in the tricarboxylic acid cycle of lesquerella embryos

Organic acids from the TCA cycle were extracted from embryos grown in medium with ^{13}C-glucose and ^{13}C-glutamine then purified for labeling analysis by LC-MS/MS.

Figure 9. (A) Metabolite purification. Metabolite extracts were purified into fractions of sugars, amino acids, and organic acids including phosphorylated compounds using stacked cation and anion exchange columns. **(B) Labeling analysis by LC-MS/MS.** Organic acids were analyzed by a UHPLC connected to a triple quadrupole mass spectrometer in MRM mode, following each compound before and after collision, losing water or CO_2.



Ex. citrate transition:			
	m_0	191	173
	m_{+1}	192	174
	...	...	...
	m_{+6}	197	179

RESULTS

Following labeling in the tricarboxylic acid cycle of lesquerella embryos

LC-MS/MS analysis of labeled organic acids resulted in parent/daughter ion values that were measured relative to one another to determine the distribution of labeled carbon in six key compounds making up the TCA cycle.

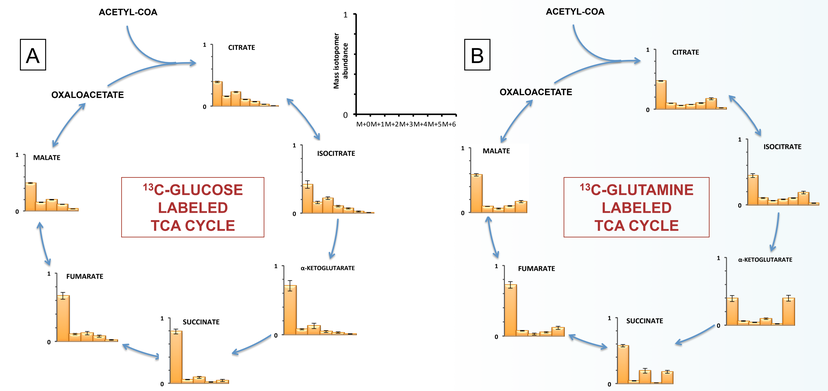


Figure 10. (A) ^{13}C-glucose labeled TCA cycle. Using ^{13}C-Glc as the labeling substrate in the lesquerella culture medium shows up in the TCA cycle as an abundance of the m+2 isotopomer, since both carbons of acetyl CoA get labeled. α-ketoglutarate through fumarate are thought to be rich in M+0 isotopomer because of the passage of unlabeled glutamate through α-KG. M+0 abundance in malate is thought to decrease due malic enzyme activity.

(B) ^{13}C-glutamine labeled TCA cycle. Using [U-^{13}C]Gln as the labeling substrate in the lesquerella culture medium shows up in α-ketoglutarate as the M+5 isotopomer. This entry into the TCA cycle is significant as M+5 labeling is more abundant than M+0 in α-ketoglutarate and remains significant for all the other organic acids. M+6 for [iso]citrate was only a small percentage.

CONCLUSION & FUTURE PLANS

• **Our results show that we have found a suitable medium for growing lesquerella *in vivo* with labeled substrates for analysis to determine targets for increasing fatty acid yield.**

• **Carbon conversion efficiency tells us to what extent a plant's intermediate metabolites end up in its biomass. Lesquerella CCE, calculated to be 85%, is much greater than that of sunflower, 50%, and on par with rapeseed which can be between to 85-95% (Alonso et al. 2007 and references therein).**

• **The synthesis of plant oils depends on huge fluxes through central metabolism. The organic acids labeling data obtained by LC-MS/MS can be used in conjunction with further analysis of labeling in sugars, phosphorylated compounds, and amino acids to build a metabolic flux map. The differences in mass isotopomer abundances between unequal neighboring compounds can be explained by linked metabolic pathways and will therefore allow the calculation of all the fluxes in primary metabolism using mathematical modeling. The flux map created can identify the sources of carbon, energy, and reductant for fatty acid synthesis.**